Kidney International, Vol. 65 (2004), pp. 1180–1190

Gene expression of adenosine receptors along the nephron

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Gene expression of adenosine receptors along the nephron.

Background. In view of the multiple effects of adenosine on kidney function, this study aimed to determine the expression of adenosine receptors (AR) along the rat and mouse nephron.

Methods. For this purpose, we semiquantified mRNA abundance for adenosine A1-, A2A-, A2B-, and A3 receptors by RNAse protection and by reverse transcription-polymerase chain reaction (RT-PCR) in the kidney zones and in the different nephron segments of mice and rats.

Results. We found very similar expression patterns for rat and mice. For the kidney zones A1-AR mRNA and A2A-AR mRNA abundance displayed a marked difference, with an increase from cortex to the inner medulla. This was not seen for A2B receptors, which showed in general a rather weak expression. Along the nephron, A1-AR was strongly expressed in the thin limbs of Henle and in the collecting duct system and to a lesser extent in the medullary thick ascending limb. A2A-AR mRNA was clearly detected in glomeruli but not in other nephron segments. A2B-AR mRNA was strongly expressed in the cortical thick ascending limb of Henle and in the distal convoluted tubule. A3-AR mRNA was not found in any nephron segment.

Conclusion. Our data demonstrate a distinct mutual expression of the AR subtypes along the nephron. A1 receptors are expressed in medullary tubular structures, while A2B receptors are predominant in cortical tubular structures. A2A receptor expression in the kidney appears to be restricted to vascular cells.

A board of evidence has accumulated that adenosine is a relevant local regulator of kidney function. Thus, adenosine modulates afferent and efferent arteriolar tone [1–3] and affects glomerular filtration [4, 5]. Adenosine furthermore influences medullary vasa recta and consequently medullary blood flow [6–10]. Adenosine affects renin secretion from renal juxtaglomerular cells [11–14] and is considered to be a signaling molecule for the tubular glomerular feedback [3, 15]. Moreover, adenosine influences tubular transport [16–20] and A1-adenosine receptor (AR) antagonists may serve as potent, natriuretic, and potassium-sparing diurectis [21, 22]. In the collecting duct, adenosine antagonizes the effects of arginine vasopressin [23]. Accumulating evidence, also, suggests that adenosine may also antagonize detrimental inflammatory events in the course of ischemic renal failure [24, 25].

The effects of adenosine are mediated by specific receptors such as A1-AR, A2A-AR, A2B-AR, and A3-AR [26]. Several studies have previously addressed partial aspects of the intrarenal localization of these adenosine receptors. These studies agree that A1-AR are expressed in the preglomerular vessels, in the juxtaglomerular apparatus, in the medullary collecting ducts [2, 27–29], and in descending vasa recta of the outer medulla [30]. A2A-AR appear to be mainly associated with renal blood vessels [2, 30], A2B-AR was found in preglomerular vessels and in descending vasa recta [29, 30], while A3-AR seem to be barely detectable in the kidney [9, 29]. However, there is still a substantial lack of knowledge about the precise intrarenal localization of adenosine receptors, particularly with regard to A2A-AR, A2B-AR, and A3-AR. Thus, functional data suggest that A1 receptor antagonist inhibit proximal tubular transport [21], while there exists not yet a convincing demonstration of A1 receptor expression in the proximal tubule [27, 28]. Furthermore, it has been reported that adenosine alters ion transport in thick ascending limb of Henle's loop [31]. For microdissected medullary thick ascending loops of Henle a strong A1-AR mRNA signal was reported [32], while in situ hybridization and immunohistochemistry provided no evidence for an expression of A1-AR mRNA there [27]. There is functional evidence for A2A receptors in preglomerular vessels [2], while neither A2A-AR protein nor mRNA are found in preglomerular vessels [27, 29]. On the other hand, in the whole kidney cortex, A2A receptor expression is well detectable [9, 29], raising the question, where else in the cortex they might be expressed. Among the different kidney zones the highest expression of A2A-AR mRNA has been reported for the inner medulla [27], while A2A receptor protein was previously demonstrated in the cortex and only a weak protein abundance was found in the medulla [29].

Key words: adenosine, nephron segments.

Received for publication June 30, 2003
and in revised form September 9, 2003, and October 8, 2003
Accepted for publication November 10, 2003

Table. 1. Sequence of primers used for reverse transcription-polymerase chain reaction (RT-PCR)

	GenBank accession number	Upstream downstream primer	Size of PCR product
β-actin	NM07393	5′-CGGGATCCCCGCCCTAGGCACCAGGGT-3′ 5′-GGAATTCGGCTGGGGTGTTGAAGGTCTCAAA-3′	286 bp
A1 mouse	GI:4884857	5′-CGGGATCCTACATCTCGGCCTTCCAGG-3′ 5′-GGAATTCAGTAGGTCTGTGGCCCAATG-3′	219 bp
A1 rat	GI:2906014	5′-CGGGATCCTACATCTCGGCCTTCCAGG-3′ 5′-GGAATTCAGTAGGTCTGTGGCCCAATG-3′	219 bp
A2A mouse	GI:2347037	5′-CGGGATCCGTCCCTGGCCATCATCGT-3′ 5′-GGAATTCGATCCTGTAGGCGTAGAT-3′	177 bp
A2A rat	GI:7407132	5′-CGGGATCCGTCCCTGGCCATCATCGT-3′ 5′-GGAATTCCCCTCAGTGCTGTGAGC-3′	293 bp
A2B mouse	GI:6680655	5′-CGGGATCCTTTCACGGCTGCCTCTTC-3′ 5′-GGAATTCCATCCCCCAGTTCTGTGC-3′	256 bp
A2B rat	GI:202587	5′-CGGGATCCAGCAGGACGCGCTGTACG-3′ 5′-GGAATTCGGAGTCAGTCCAATGCCAAAG-3′	406 bp
A3 mouse	GI:25020505	5′-CGGGATCCCGTTCCGTGGTCAGTTTG-3′ 5′-GGAATTCGCAGGCGTAGACAATAGG-3′	348 bp
A3 rat	GI:6978452	5′-CGGGATCCGCTGTAGTGGGCAACATGC-3′ 5′-GGAATTCGATGATGCACATGACAACCAG-3′	563 bp

In view of the important renal effects of adenosine and the existing uncertainties regarding the intrarenal localization of adenosine receptor expression, our study aimed to systematically semiquantitate adenosine receptor gene expression in the different nephron segments. To narrow down species-specific expression, we performed our analysis in parallel in rat and mouse tissue.

METHODS

Animals

Animal experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the care and use of laboratory animals and the German laws on the protection of animals. Male C57bl/6 mice (20 to 24 g) and male Sprague-Dawley rats (230 to 270 g), which had free access to tap water and standard commercial pellet chow (Altromin C-1000; Altromin, Lage, Germany) were used.

Organ sampling and RNA extraction

Animals were killed by decapitation and kidneys were immediately removed and cooled in 4°C cold minimal essential medium (MEM). The kidneys were carefully separated in cortex and outer and inner medulla under a stereomicroscope. The separated tissue was frozen in liquid nitrogen. Total RNA was extracted from frozen kidney zones stored at −80°C according to the protocol of Chomczynski and Sacchi [33]. After homogenization in guanidine thiocyanate solution [4 mol/L, containing 0.5% *N*-lauryl-sarcosinate, 10 mmol/L ethylenediaminetetraacetic acid (EDTA), 25 mmol/L sodium citrate, and 700 mmol/L β-mercaptoethanol], 0.1 volume 2 mol/L sodium acetate (pH 4), 1 volume phenol (water saturated), and 0.2 volume chloroform were added sequentially to the homogenate. After cooling on ice for 15 minutes, samples were centrifuged at 10,000*g* for 20 minutes at 4°C. RNA in the supernatant was precipitated with an equal volume of isopropanol at −20°C for at least 1 hour. The resulting RNA pellets were resuspended in guanidine thiocyanate solution, and again precipitated with an equal volume of isopropanol at −20°C. After washing the RNA pellets with 70% ethanol (EtOH) the RNA was dissolved in diethylpyrocarbonate-treated water, and stored at −80°C until further processing. The yield of RNA was measured at 260 nm.

RNase protection assay

The mRNA levels of the adenosine receptors A1-AR, A2A-AR, and A2B-AR in different kidney zones were measured by RNase protection assay. The A1-AR specific probe covers 219 bp, the A2A-AR probe 122 bp, and the A2B-AR probe 406 bp plus a 60 bp polylinker sequence of the vector. In addition, β-actin was assayed by RNase protection in all RNA samples as a housekeeping gene product to obtain an internal control of RNA quality.

For construction of the plasmid vectors, the fragments of A1-AR, A2A-AR, and A2B-AR were amplified (primers given in Table 1) with cDNA of rat kidney as template in the polymerase chain reaction (PCR). The amplified fragments were verified on a 2% agarose gel, purified, and digested with *Bam*H1 and *Eco*RI (Pharmacia Biotech, Uppsala, Sweden). After separation on a 2% agarose gel the DNA was extracted (QiaexII) (Quiagen, Hilden, Germany) and ligated for 12 hours at 14°C into *Bam*H1/*Eco*RI polylinker sites of vector psp73 (Promega, Madison, WI, USA) for heat shock uptake into *Escherichia coli* (JM109) (Promega). Due to a *Bam*H1 site in the 239 bp A2A-AR PCR fragment the resulting DNA ligated into the psp73 vector covered only 122 bp. Positive clones were grown and plasmids were isolated (Maxi-Kit) (Quiagen). Accuracy of the

constructed plasmids was conformed by a sequence analysis performed by Sequiserve (Vaterstetten, Germany). Linearization of the plasmid vectors with *Hin*dIII was followed by in vitro transcription with SP6 polymerase (Promega) in the presence of α^{32}P-guanosine triphosphate (GTP) (Amersham, Buckinghamshire, UK). The radiolabeled antisense cRNA probes were hybridized with total RNA in solution at 60°C for 14 to 18 hours, then digested with RNase A/T1 [reverse transcription (RT) for 30 minutes), and proteinase K added (37°C for 30 minutes). After phenol/chloroform extraction and ethanol precipitation, the protected probes were separated by electrophoresis on 8% polyacrylamide gels. The amount of radioactivity in the hybrids was assessed by a β-counter and autoradiography was performed at −80°C.

Real-time PCR analysis

Two micrograms of whole kidney, cortex, outer, and inner medulla total RNA or resulting RNA pellets of the microdissected tubules were reverse transcribed into cDNA (20 μL) according to standard protocols. Real-time PCR was performed in a Light Cycler (Roche, Mannheim, Germany). All experiments were done using the Light Cycler DNA SYBR Green I Kit (Roche). Each reaction contained 2 μL Master Mix [including Taq polymerase, desoxynucleoside triphosphates (dTNPs), SYBR Green, buffer], 1 pmol of each primer (Table 1), 3 mmol/L (β-actin, A1-AR, and A2A-AR, and rat A3-AR) or 5 mmol/L $MgCl_2$ (A2B-AR, mouse A3-AR), and 2 μL cDNA. After 10 minutes at 94°C for activation of the Taq polymerase, the amplification program consisting of 15 seconds at 94°C, 10 seconds at 60°C, and 20 seconds at 72°C was performed for 50 cycles. The amplification was followed by a melting curve analysis for control of the PCR products. As negative controls water and yeast tRNA instead of cDNA was run with every PCR experiment. To verify the accuracy of the amplification, PCR products were further analyzed on ethidium bromide stained 2% agarose gels. Analysis of the data was performed using Light Cycler software 3.5.3. Standard curves for adenosine receptor mRNAs and β-actin mRNA were produced by using cDNA of whole kidney or cortex, which was diluted 1:5, 1:10, 1:50, 1:100, and 1:1000. The ratio of the amount of adenosine receptor mRNA and β-actin mRNA was calculated for each sample.

Microdissection of nephron segments and RT-PCR analysis

Nephron segments for RT-PCR and real-time PCR were obtained by a modified collagenase digestion protocol developed by Schafer et al [34] and described by us in detail elsewhere [35]. Collecting of the nephron segments was carried out separately in cortex, outer, and inner medulla over a time period of 3 to 4 hours after digesting. For collecting thin limb (dTL/aTL) and inner medullary collecting ducts (IMCD), the digested pieces of the inner medulla were further separated with sharpened forceps. The differentiated collected tubules were separately transferred to a culture dish containing 0.25% bovine sersum albumin (BSA) solution in MEM. The nephron segments were measured and at least 11 mm of each segment [proximal convoluted tubule (PCT), proximal straight tubule (PST), outer medullary descending thin limb of Henle's loop (dTL), inner medullary descending and ascending thin limb (dTL/aTL), medullary and cortical thick ascending limb of Henle's loop (mTAL and cTAL, respectively], distal convoluted tubule (DCT), connecting tubule and cortical collecting duct (CT/CCD), outer medullary and inner medullary collecting duct (OMCD and IMCD, respectively) were pooled. In addition, at least 22 glomeruli with and without arterioles attached ($Glom^+$ and $Glom^-$, respectively) were collected. Collected tubules were transferred to 400 μL GTC solution (guanidine thiocyanate (4 mol/L) containing 0.5% *N*-lauryl-sarcosinate, 10 mmol/L EDTA, 25 mmol/L sodium citrate, 700 mmol/L β-mercaptoethanol) and stored at −80°C until RNA was extracted. Total RNA was extracted according to the protocol of Chomczynski and Sacchi [33]. In brief, after defrosting the samples 12 μg yeast tRNA, 0.1 volume 2 mol/L sodium acetate (pH 4), 1 volume phenol (water saturated), and 0.2 volume chloroform were added sequentially to the homogenate. After cooling on ice for 15 minutes, samples were centrifuged at 10,000*g* for 20 minutes at 4°C. RNA in the supernatant (360 μL) was precipitated with an equal volume of isopropanol at −20°C for at least 1 hour. The resulting RNA pellets were washed with 70% EtOH and finally dissolved in 9 μL diethylpyrocarbonate-treated water and used for RT using standard RT protocol. Resulting cDNA samples were diluted to a final concentration of 1 mm tubules/2 μL and two glomeruli/2 μL. PCRs were performed in a total volume of 20 μL in the presence of 2 μL cDNA equivalent to 1 mm tubule or two glomeruli. Negative controls included water instead of cDNA in the PCR, and yeast tRNA (carrier used for RNA isolation) with no further addition of RNA in the RT. As positive controls for the PCR, cDNA of 0.2 μg whole kidney RNA were amplified in all experiments. PCR was run with 30 seconds/94°C denaturation, 30 seconds/60°C annealing, and 1 minute/72°C extension using standard PCR protocols. PCR amplification was done with primers given in Table 1. Analysis of PCR products was performed after size separation by ethidium bromide-stained 2% agarose gel electrophoresis. For control of RNA isolation and RT, PCR with β-actin primers (30 cycles) were performed. In all samples, β-actin was equally amplified with cDNA equivalent to 1 mm tubule length or two glomeruli.

PCR of all adenosine receptors were performed on the cDNA derived from at least three different sets of nephron segments from different rats and mice. Each set always included Glom, PCT, PST, dTL, dTL/aTL, mTAL, cTAL, DCT, CT/CCD, OMCD, and IMCD. In rat tissue, vasa recta of the outer medulla (VROM) were also collected.

RESULTS

Zonal distribution of A1-AR, A2A-AR, and A2B-AR mRNA

Expression of A1-AR, A2A-AR, and A2B-AR mRNA was analyzed by RNase protection assay in total RNA of kidneys of six rats (Fig. 1). All three receptor mRNAs were detected in cortex and outer and inner medulla. The A2A-AR showed the highest overall mRNA expression level in the whole kidney, followed by A1-AR mRNA and A2B-AR mRNA abundance. The ratio of the overall abundance of A1-AR, A2A-AR, and A2B-AR mRNA in the whole rat kidney was 1:2.6:0.6. The expression of the A3-AR mRNA could be detected only by RT-PCR with a cycle number higher than 32.

The intrarenal distribution of the A1-AR, A2A-AR, and A2B-AR gene expression was determined by RNase protection assay in the different kidney zones, namely, cortex and outer and inner medulla, in six rats. The expression of the A1-AR was highest in the inner medulla and lowest in the cortex (Fig. 1). The relative expression level (normalized to cortex) for the A1-AR mRNA abundance was 1:6:29 for cortex, outer medulla, and inner medulla, respectively. The expression of the A2A-AR gene was also the highest in the inner medulla and lowest in the cortex yielding a ratio of 1:4:10 for cortex, outer medulla, and inner medulla, respectively. The expression of the A2B-AR gene was rather weak in all kidney zones. The interzonal ratio of A2B-AR mRNA was 1:1.7:2.0, for cortex, outer medulla, and inner medulla, respectively.

We also determined the interzonal expression of the adenosine receptor mRNAs by real-time PCR for further validation of the method (Fig. 2). Using total RNA of four out of the six animals analyzed in Figure 1, we found for A1-AR mRNA a ratio of 1:4:27, for A2A-AR mRNA a ratio of 1:3:11, and for A2B-AR mRNA a ratio of 1:1.2:1.2 for cortex, outer medulla, and inner medulla, respectively. As these data were very similar to that obtained by RNAse protection, we considered real-time PCR as a robust method to semiquantitate mRNA in smaller samples of total RNA.

Distribution of the adenosine receptor mRNAs in nephron segments

The localization of the A1-AR mRNA, A2A-AR mRNA, A2B-AR mRNA, and A3-AR mRNA along the

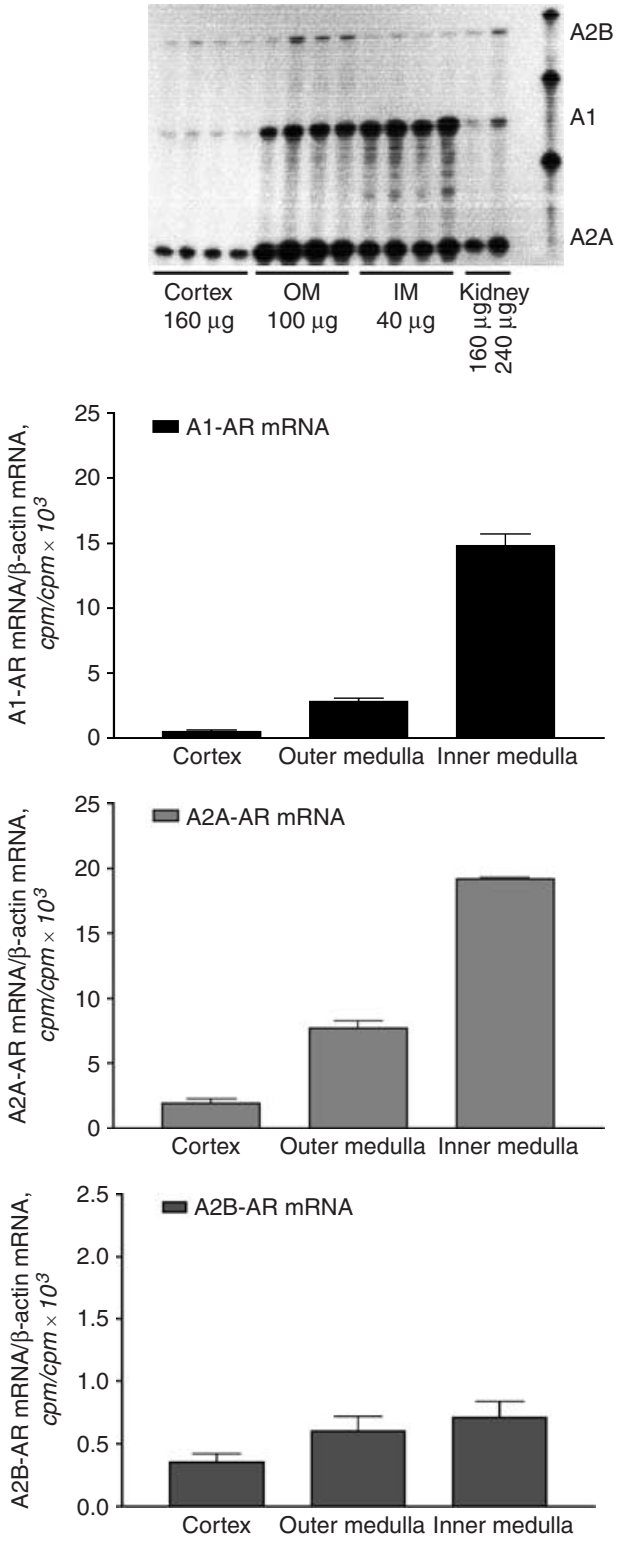

Fig. 1. Abundance of A1-AR mRNA, A2A-AR mRNA, and A2B-AR mRNA in the rat kidney zones cortex, outer medulla (OM) and inner medulla (IM) relative to β-actin mRNA levels. The expression level of A1-AR mRNA (*A*) and A2A-AR mRNA (*B*) was highest in the inner medulla, whereas A2B-AR (*C*) was distributed equally in the three zones (mean ± SEM; N = 6 each). *Inset* shows autoradiographs of a representative RNAse protection assay with P^{32}-labeled A2B receptor (A2B), A1 receptor (A1), and A2A receptor (A2A) probes.

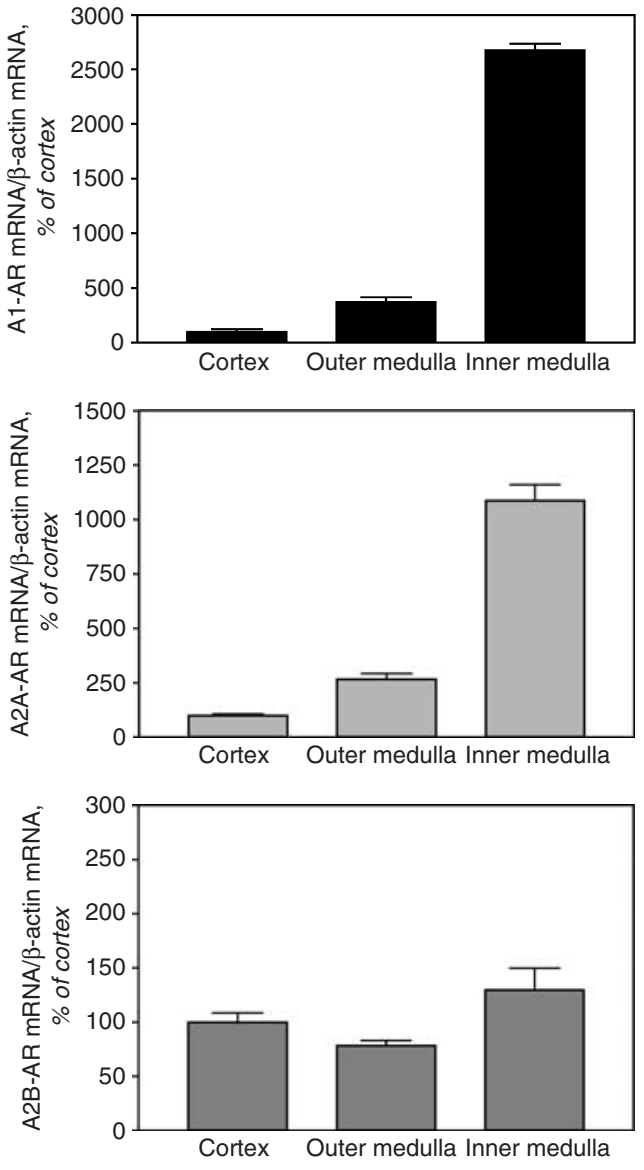

Fig. 2. Expression profile of the A1-AR mRNA, A2A-AR, and A2B-AR mRNA in rat kidney cortex, outer and inner medulla relative to β-actin mRNA determined by real-time reverse transcription-polymerase chain reaction (RT-PCR) (mean ± SEM; $N = 4$ each).

rat and mouse nephron was investigated by RT-PCR. Each receptor mRNA was tested at least on three different pools of nephron segments, including glomeruli (Glom), PCT and PST, dTL and dTL/aTL, mTAL and cTAL, DCT, CT/CCD, and OMCD and IMCD. Each pool contained material from three to five animals.

A1-AR mRNA localization

In microdissected segments A1-AR mRNA (Fig. 3A) was strongly expressed in dTL and dTL/aTL and OMCD and IMCD both in rats and mice. A weaker A1-AR mRNA abundance was found in CT/CCD, mTAL, cTAL, and DCT and in glomeruli with and without arterioles ($Glom^+$ and $Glom^-$, respectively). Only a very faint signal was obtained for PCT and PST. The localization of A1-AR mRNA was confirmed in four and three different pools, respectively, of collected nephron segments of rats and mice.

Since the juxtaglomerular portion of afferent arterioles is considered as a main target of A1-AR action, the moderate expression of A1-AR mRNA in glomeruli was somewhat unexpected. We therefore semiquantitated A1-AR mRNA by real-time PCR in three different pools of microdissected glomeruli either with ($Glom^+$) or without ($Glom^-$) visible attached afferent arterioles, each pool comprising 80 glomeruli. For comparison, the A1-AR mRNA abundance (A1-AR mRNA/β-actin mRNA) in the glomeruli pools was set into proportion with the A1-AR mRNA abundance in the whole kidney (i.e., 100%).

In rats, the pools of $Glom^+$ and $Glom^-$ contained on average an A1-AR mRNA abundance of 56% and 21% of the whole kidney, respectively. The respective values for mouse kidneys were 23% for $Glom^+$ and 10% for $Glom^-$ (Fig. 3B). These data suggest that the afferent arteriole rather than the glomerulus expresses A1-AR mRNA and that the expression level there is below the average of the whole kidney.

A2A-AR mRNA localization

RT-PCR for A2A-AR mRNA was performed with four rat and three mouse pools of microdissected nephron segments. The PCR product was always found in the whole kidney and in the glomeruli, while all other nephron segments produced only faint bands (Fig. 4A). Even in glomeruli, the A2A-AR mRNA level appeared relatively low when compared with the expression in the whole kidney. The relative abundance of glomerular A2A-AR mRNA related to mouse cortex as measured by real-time PCR in three different pools of microdissected glomeruli either with ($Glom^+$) or without ($Glom^-$) visible attached afferent arterioles revealed a 3.5-fold enrichment of A2A-AR mRNA in glomeruli relative to the cortex (Fig. 4B). There was no difference in the expression level of A2A-AR mRNA between glomeruli with or without attached afferent arteriole (Fig. 4).

A2B-AR mRNA localization

RT-PCR for A2B-AR mRNA was performed with four rat and three mouse pools of microdissected nephron segments. In both species the A2B-AR transcript was detected in the distal tubules (Fig. 5). In rats, the expression in the distal tubules was restricted to the cTAL and DCT. A weak expression was furthermore found in CT/CCD. In mice RT-PCR of the A2B-AR mRNA revealed the

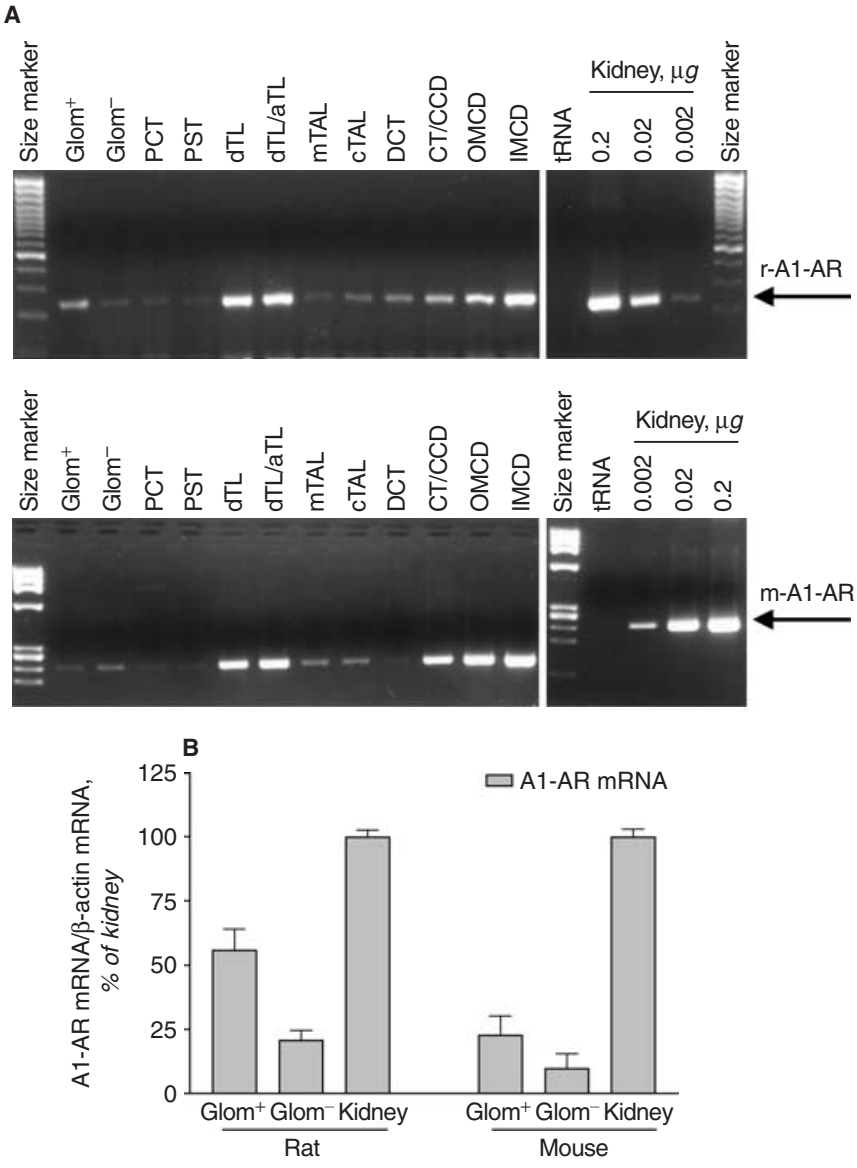

Fig. 3. (*A*) Nephron distribution of adenosine receptor A1-AR mRNA in microdissected nephron segments of the rat (r-A1-AR) and mouse (m-A1-AR) kidney. Representative ethidium bromide-stained agarose gel displaying the distribution of the A1-AR receptor mRNA after reverse transcription-polymerase chain reaction (RT-PCR) (rat 34 cycles; mouse 36 cycles). (*B*) Expression level of the A1-AR mRNA relative to β-actin mRNA in microdissected glomeruli of rat and mouse kidney assayed by real time PCR ($N = 3$ each). The A1-AR mRNA/β-actin mRNA level in the glomeruli was compared mRNA level of the whole kidney (100%) of the rat and mouse, respectively. Abbreviations are: $Glom^+$, glomeruli with arterioles; $Glom^-$, glomeruli without arterioles attached; PCT, proximal convoluted tubule; PST, proximal straight tubule; dTL, outer medullary descending thin limb of Henle's loop; dTL/aTL, inner medullary descending and ascending thin limb of Henle's loop; mTAL, medullary thick ascending limb of Henle's loop; cTALH, cortical thick ascending limb of Henle's loop; DCT, distal convoluted tubule; CT/CCD, connecting tubule and cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; tRNA, negative control, kidney 0.2, 0.02, and 0.002 µg RNA from the whole kidney as positive control for the PCR.

strongest expression in DCT and cTAL, and in addition a weaker expression in mTAL and CT/CCD.

The expression of A2B-AR mRNA in glomeruli differed somewhat between rats and mice. In rats the expression of A2B-AR mRNA in glomeruli was always found in microdissected glomeruli. In mice the A2B-AR transcript was only occasionally observed in glomeruli at trace levels.

A3-AR mRNA localization

The overall abundance of A3-AR mRNA in the whole kidney was rather low. Although A3-AR mRNA was detected in the whole kidney with a high PCR cycle number, was the A3-AR transcript not found in any nephron segment (Fig. 6). To rule out that faint signals obtained for A3-AR mRNA in kidney was a primer related artifact, we also assayed A3-AR mRNA in rat (Fig. 6, left panel) and mouse (Fig. 6, right panel) brain, heart, and kidney. For all experiments cDNA from 0.2 µg total RNA of each organ was used. In contrast to the kidney produced brain and heart strong bands for A3-AR mRNA in both animals species, supporting the assumption that the abundance of A3-AR mRNA in the kidney in fact is very low.

Distribution of the adenosine receptor mRNA in VROM

To determine the expression of adenosine receptors in the medullary blood vessels we sampled VROM of rats. cDNA derived from nine pools of bundles of vessels ($N = 9$) was assayed for A1-AR, A2A-AR, A2B-AR, and A3-AR mRNA. Representative results are shown in Figure 7. All VROM of rat pools were clearly positive for A1-AR mRNA and A2A-AR mRNA and to a lesser

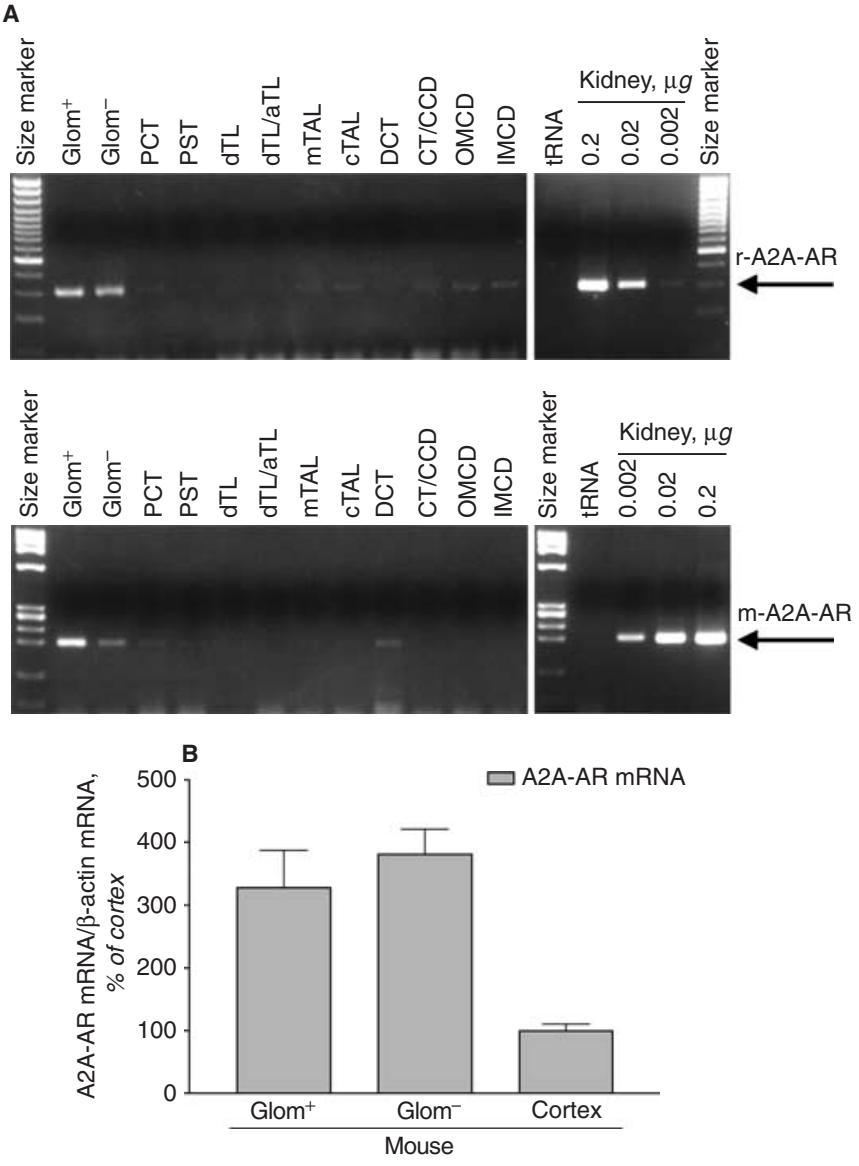

Fig. 4. (*A*) Nephron distribution of adenosine receptor A2A mRNA in microdissected nephron segments of the rat (r-A2A-AR) and mouse (m-A2A-AR) kidney. The distribution of A2A-AR mRNA after reverse transcription-polymerase chain reaction (RT-PCR) with 32 cycles (rat) and 36 cycles (mouse) is shown by an ethidium bromide-stained agarose gel. (*B*) Expression level of the A2A-AR mRNA/β-actin mRNA in mouse glomeruli compared to the expression level in the rat cortex (100%). Abbreviations are: $Glom^+$, glomeruli with arterioles; $Glom^-$, glomeruli without arterioles attached; PCT, proximal convoluted tubule; PST, proximal straight tubule; dTL, outer medullary descending thin limb of Henle's loop; dTL/aTL, inner medullary descending and ascending thin limb of Henle's loop; mTAL, medullary thick ascending limb of Henle's loop; cTALH, cortical thick ascending limb of Henle's loop; DCT, distal convoluted tubule; CT/CCD, connecting tubule and cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; tRNA, negative control, kidney 0.2, 0.02, and 0.002 µg RNA from the whole kidney as positive control for the PCR.

extent also for A2B-AR mRNA. mRNA of the A3-AR was not detected in any VROM in rat pool.

DISCUSSION

In our study on the gene expression of adenosine receptors in the rat and mouse kidney we found an overall expression level of A2A-AR mRNA > A1-AR mRNA >> A2B-AR mRNA >> A3-AR mRNA. This may not necessarily reflect protein abundance but may be indicative for the functional relevance of the respective receptors in the kidney.

For the zonal A1-AR gene expression we found a strong corticomedullary uphill gradient. This is in good agreement with previous data obtained by in situ hybridization [27]. The zonal distribution results from the distinct expression pattern of the A1-AR gene along the nephron, which showed marked corticomedullary uphill gradients for the descending loop of Henle, for the ascending loop of Henle and for the collecting duct system. In the cortex, we also found a significant expression of the A1-AR gene in glomeruli in association with afferent arterioles, while proximal tubules as the main mass of the cortex were negative for A1-AR mRNA in rats and mice. A rather similar expression pattern of the A1-AR gene was previously reported by Weaver and Reppert [27] and in part by Yamaguchi et al [32], except the strong expression in the thin limbs of Henle, which is for the time described in this study. In accordance with the study by Weaver and Reppert [27] but in contrast to the report by Yamaguchi et al [32], we found no strong expression of A1-AR mRNA in the thick ascending limb

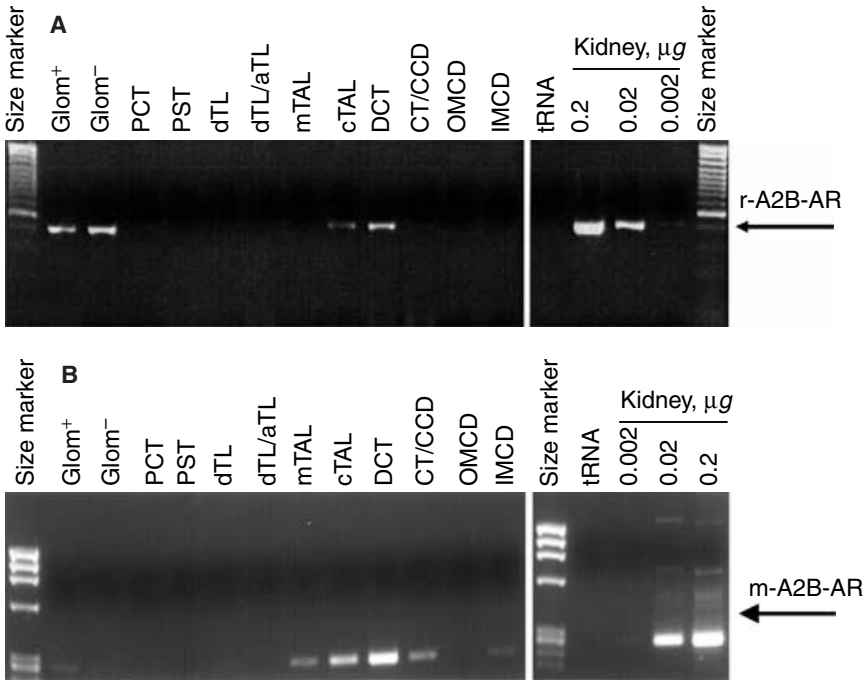

Fig. 5. Nephron distribution of adenosine receptor A2B-AR mRNA in microdissected nephron segments of the rat (r-A2B-AR) and mouse (m-A2B-AR) kidney. Distribution of A2B-AR mRNA after reverse transcription-polymerase chain reaction (RT-PCR) with 36 cycles in rat (*A*) and mouse (*B*) shown by an ethidium bromide-stained agarose gel. Abbreviations are: $Glom^+$, glomeruli with arterioles; $Glom^-$, glomeruli without arterioles attached; PCT, proximal convoluted tubule; PST, proximal straight tubule; dTL, outer medullary descending thin limb of Henle's loop; dTL/aTL, inner medullary descending and ascending thin limb of Henle's loop; mTAL, medullary thick ascending limb of Henle's loop; cTALH, cortical thick ascending limb of Henle's loop; DCT, distal convoluted tubule; CT/CCD, connecting tubule and cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; tRNA, negative control, kidney 0.2, 0.02, and 0.002 µg RNA from the whole kidney as positive control for the PCR.

of Henle. In addition, our data support previous reports on the expression of A1-AR mRNA in medullary vasa recta [30].

The expression of the A1-R in glomeruli and afferent arterioles is in good agreement with a number of functional studies indicating a vasoconstriction of afferent arterioles by adenosine via A1-AR and inhibition of renin secretion from the juxtaglomerular apparatus by adenosine via A1-AR [36]. There is also evidence for a relevant function of A1-AR to inhibit sodium transport [37] and antagonize the action of antidiuretic hormone [38] in the collecting duct system, in particular in the inner medullary collecting duct. Rather contradictory are findings about the existence of A1-AR in proximal tubules. While functional studies suggest a strong natriuretic and diuretic effect of A1-AR antagonists on proximal tubules [21, 39, 40] we and others [27, 32] could not detect significant A1-AR mRNA levels in this particular nephron segment.

Our novel results showing the strong expression of the A1-AR gene in the thin limbs of Henle await functional explanation. If water resorption and sodium chloride resorption are the main functions of the descending and ascending thin limbs, respectively, one may speculate that adenosine via A1-AR affects these functions. Given that A1-AR signals through inhibition of cyclic adenosine monophosphate (cAMP) formation and through elevation of cytosolic calcium [41], it remains to be clarified how these second messengers determine the function of the thin loops of Henle.

As for A1-AR, we also found for the expression of the A2A-AR gene a strong corticomedullary uphill gradient. This is in accordance with a previous report [27]. Furthermore, the clear signals obtained for A2A-AR mRNA in glomeruli with and without visible attached arterioles and in microdissected vasa recta are in principle compatible with previous conclusions and assumptions about the expression of the A2A-AR in afferent arterioles and in vasa recta [2, 8, 27, 29, 30]. Considering the similar abundance of A2A-AR mRNA in glomeruli with and without afferent arteriole ($Glom^+$ and $Glom^-$), suggests that intraglomerular cells are a main site of A2A expression and that A2A receptors are not enriched in afferent arterioles relative to glomeruli. In analogy to our findings, the existence of A2A-AR in outer medullary descending vasa recta (OMVR) in the rat was also previously shown [8, 30].

In contrast to the vascular structures, we obtained no clear signal for A2A-AR mRNA in any tubule segment. This was to our surprise since we found a similar significant expression as of A1-AR in the inner medulla for A2A-AR mRNA. In contrast to A1-AR, neither thin limbs nor collecting ducts contained significant amounts of A2A-AR mRNA. Expression of A2A-AR in the inner medulla in nontubular structures has previously already been suggested by Weaver and Reppert [27]. By exclusion, one can therefore infer that A2A-AR in the kidney medulla is restricted to blood vessels and potentially to interstitial cells. Such a restricted expression of A2A-AR in medullary blood vessels would be in a good agreement with the concept, that A2A-AR regulates the blood circulation in the medulla [30, 42].

In contrast to A1-AR and A2A-AR we found an only low abundance of A2B-AR mRNA in the whole kidney,

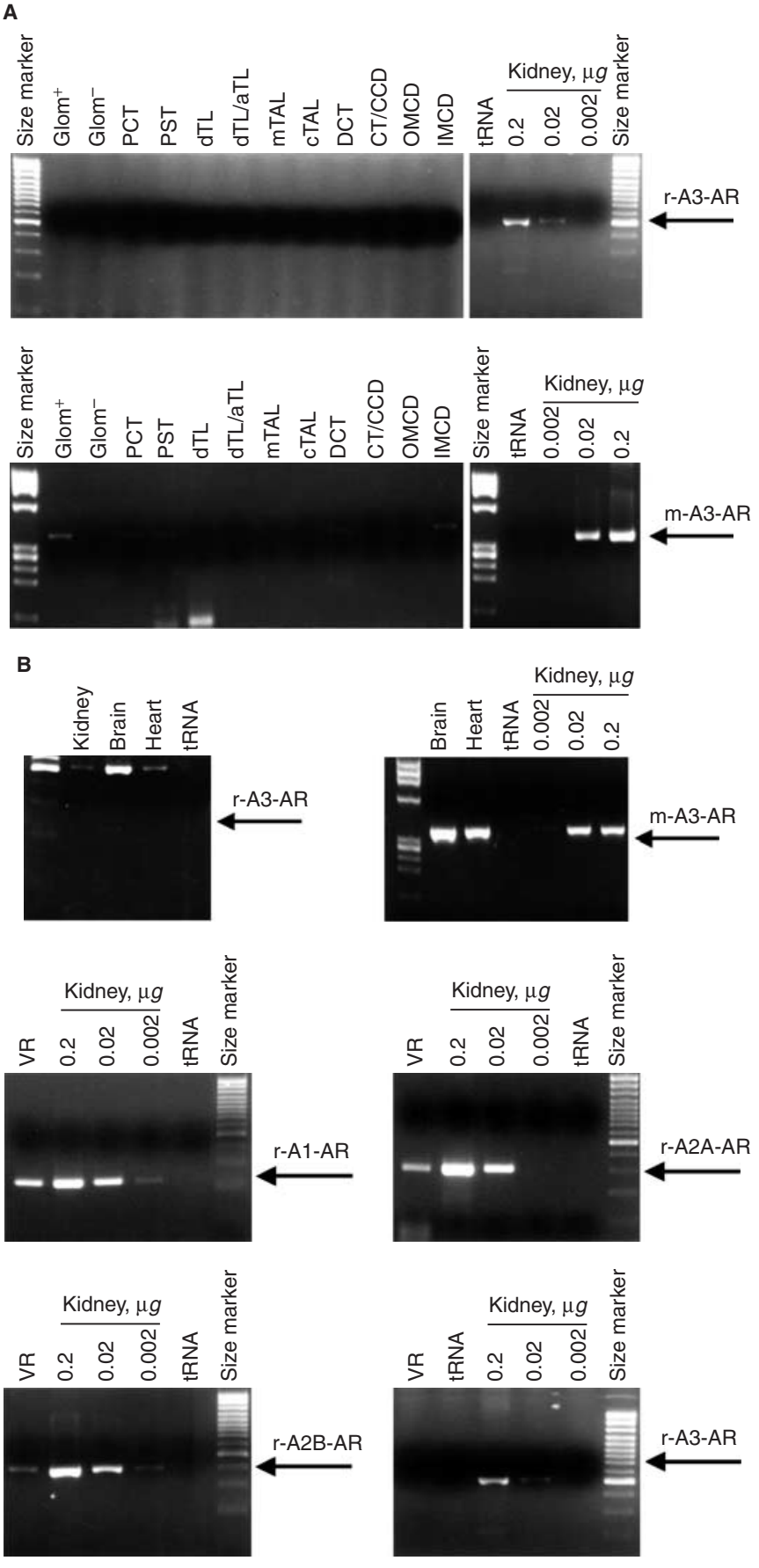

Fig. 6. (*A*) Localization of the adenosine receptor A3-AR mRNA in microdissected nephron segments of the rat (r-A3-AR) and mouse (m-A3-AR) kidney. Ethidium bromide-stained agarose gel showing the A3-AR mRNA after reverse transcription-polymerase chain reaction (RT-PCR) with 39 cycles (rat) and 36 cycles (mouse) in the kidney. (*B*) Abundance of A3-AR mRNA in the rat (left panel, 34 cycles) and mouse (right panel, 36 cycles) kidney, brain, and heart. From each organ 0.2 µg RNA was used for RT-PCR (except in mouse kidney 0.02 µg and 0.002 µg were tested in addition). Abbreviations are: $Glom^+$, glomeruli with arterioles; $Glom^-$, glomeruli without arterioles attached; PCT, proximal convoluted tubule; PST, proximal straight tubule; dTL, outer medullary descending thin limb of Henle's loop; dTL/aTL, inner medullary descending and ascending thin limb of Henle's loop; mTAL, medullary thick ascending limb of Henle's loop; cTALH, cortical thick ascending limb of Henle's loop; DCT, distal convoluted tubule; CT/CCD, connecting tubule and cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; tRNA, negative control, kidney 0.2, 0.02, and 0.002 µg RNA from the whole kidney as positive control for the PCR.

Fig. 7. Expression of adenosine receptor A1, A2A, A2B, and A3 mRNA in vasa recta from the outer medulla of the rat (VR). Adenosine receptor mRNA after reverse transcription-polymerase chain reaction (RT-PCR) (A1-AR, 34 cycles; A2A-AR, 32 cycles; A2B-AR, 36 cycles; and A3-AR, 39 cycles). TRNA is negative control, kidney: 0.2, 0.02, and 0.002 µg RNA from the whole kidney as positive control for the PCR.

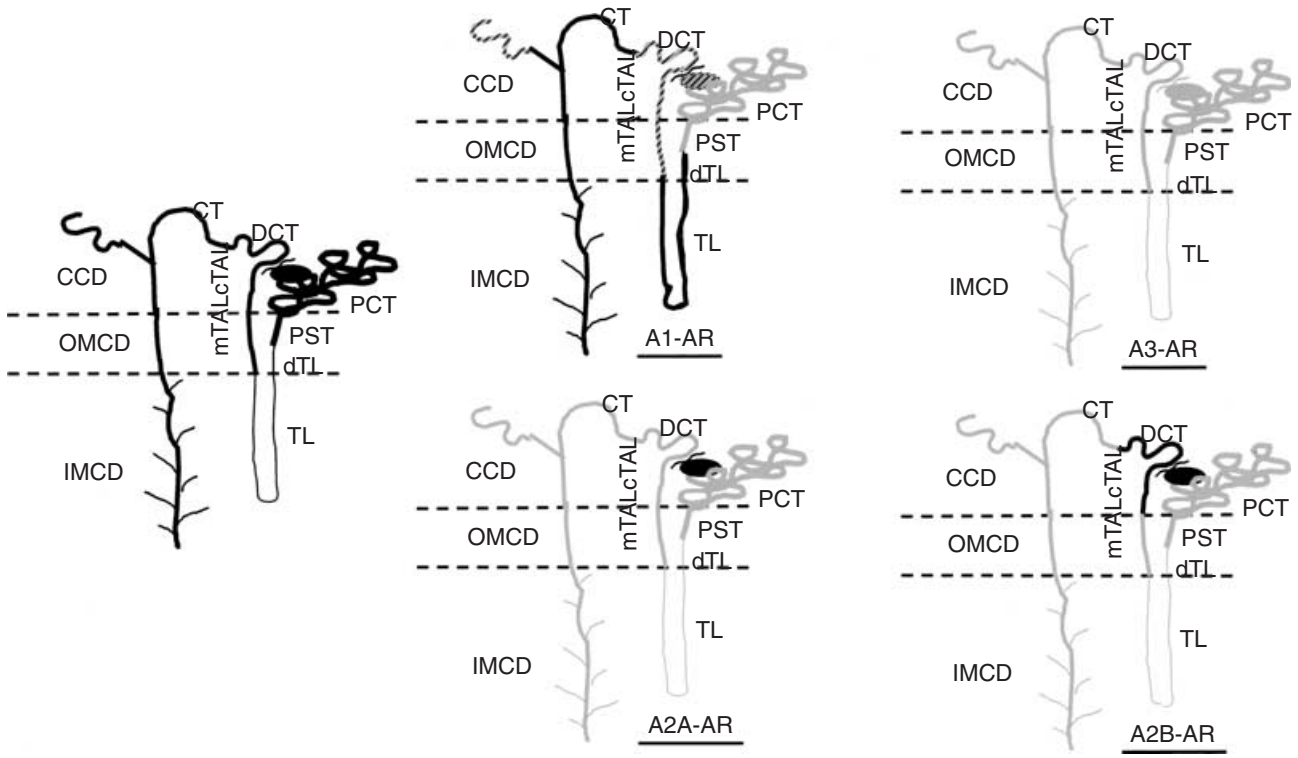

Fig. 8. Schematic illustration of the gene expression of adenosine receptors A1-AR, A2A-AR, A2B-AR, and A3-AR in the nephron of the rat kidney. The left figure gives an overview about the different nephron segment. A1-mRNA is strongly expressed in thin limbs of loop of Henle and in the collecting duct system and more weakly expressed in glomerulus/afferent arteriole as well as in the thick ascending limb of loop of Henle. A2A-mRNA is clearly expressed in glomerulus/afferent arteriole. A2B-mRNA is mainly found in the cortical thick ascending limb of loop of Henle, in the distal convoluted tubule and somewhat less in glomerulus/afferent arteriole. A3-mRNA is not found in any segment. Not indicated in the figure is the expression of A1-, A2A- and A2B- mRNA in medullary vasa recta. Abbreviations are: $Glom^{+}$, glomeruli with arterioles; $Glom^{-}$, glomeruli without arterioles attached; PCT, proximal convoluted tubule; PST, proximal straight tubule; dTL, outer medullary descending thin limb of Henle's loop; dTL/aTL, inner medullary descending and ascending thin limb of Henle's loop; mTAL, medullary thick ascending limb of Henle's loop; cTALH, cortical thick ascending limb of Henle's loop; DCT, distal convoluted tubule; CT/CCD, connecting tubule and cortical collecting duct; OMCD, outer medullary collecting duct; IMCD, inner medullary collecting duct; tRNA, negative control, kidney 0.2, 0.02, and 0.002 μg RNA from the whole kidney as positive control for the PCR.

similar to a previous report [27]. In addition, our data indicate that there is no clear intrarenal corticomedullary expression gradient. The nephron analysis furthermore indicates a species dependent expression of A2B-AR associated with glomeruli in rats but not in mice. In accordance with previous data we also found expression of A2B-AR in vasa recta [30]. A novel finding of our study is the significant expression of A2B-AR mRNA in the cTAL and the DCT both in rats and mice.

While the expression of A2B-AR in renal arterioles points toward a function of these receptors in the control of intrarenal blood flow [43], remains the function of A2B-AR in the distal nephron more speculative and must be determined therefore in future experiments.

The A3-AR gene was only very weakly expressed in the whole kidney and we could not find gene transcripts in tubules, glomeruli, or vasa recta. We therefore speculate that the functional relevance of A3-AR in the kidney is less than that for the other adenosine receptors. So far, little is known about the potential effects mediated by A3-AR in the kidney.

Altogether, our data show a distinct mutual expression of the adenosine receptor subtypes along the nephron. The A1 receptors are expressed in medullary tubular structures, while the A2B receptors predominate in cortical tubular structures. A2A receptor expression in the kidney appears to be restricted to vascular cells.

ACKNOWLEDGMENT

The authors thank Karl Kunzelmann for critical reading of the manuscript.

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